Biochimica et Biophysica Acta 1853 (2015) 1564–1573

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Programmed cell death 4 protein (Pdcd4) and homeodomain-interacting protein kinase 2 (Hipk2) antagonistically control translation of Hipk2 mRNA

Johanna Ohnheiser [a,1], Eva Ferlemann [a,1], Astrid Haas [a], Jan P. Müller [a], Eugen Werwein [a], Olesja Fehler [a], Abhiruchi Biyanee [a,b], Karl-Heinz Klempnauer [a,*]

[a] Institut für Biochemie, Westfälische-Wilhelms-Universität Münster, Wilhelm-Klemm-Str. 2, D-48149 Münster, Germany
[b] Graduate School of Chemistry (GSC-MS), Westfälische-Wilhelms-Universität Münster, Germany

ARTICLE INFO

Article history:
Received 12 February 2015
Received in revised form 11 March 2015
Accepted 14 March 2015
Available online 25 March 2015

Keywords:
Pdcd4
Hipk2
HIPK1
Translation
Autoregulation

ABSTRACT

The tumor suppressor protein programmed cell death 4 (Pdcd4) is a highly conserved RNA-binding protein that inhibits the translation of specific mRNAs. Here, we have identified the homeobox-interacting protein kinase-2 (Hipk2) mRNA as a novel translational target of Pdcd4. Unlike most other protein kinases Hipk2 is constitutively active after being synthesized by the ribosome and its expression and activity are thought to be mainly controlled by modulation of the half-life of the kinase. Our work provides the first evidence that Hipk2 expression is also controlled on the level of translation. We show that Hipk2 stimulates the translation of its own mRNA and that Pdcd4 suppresses the translation of Hipk2 mRNA by interfering with this auto-regulatory feedback mechanism. We also show that the translation of the related kinase Hipk1 is controlled by a similar feedback loop and that Hipk2 also stimulates the translation of Hipk1 mRNA. Taken together, our work describes a novel mechanism of translational suppression by Pdcd4 and shows for the first time that Hipk2 controls its own synthesis by an auto-regulatory feedback mechanism. Furthermore, the effect of Hipk2 on the translation of Hipk1 RNA suggests that Hipk2 and Pdcd4 can act in similar manner to control the translation of other mRNAs.

1. Introduction

Pdcd4 (*Programmed cell death 4*) was initially identified as a gene whose expression is increased during apoptosis [1] and was subsequently shown to function as a tumor suppressor in an *in-vitro* keratinocyte model of tumor promotion [2] and an *in-vivo* model of skin carcinogenesis [3]. Decreased expression of *Pdcd4* has now been implicated in the development of several human cancers, including lung, colon, liver and breast cancer and glioblastoma [4–8], further supporting its role as a tumor suppressor gene. In many cases, suppression of *Pdcd4* expression is due to increased expression of the oncogenic micro-RNA miR-21, which targets the 3′ untranslated region of *Pdcd4* mRNA [9–11]. On the protein level, Pdcd4 can be down-regulated by S6K-mediated phosphorylation which triggers its ubiquitination and subsequent degradation *via* the E3 ubiquitin ligase complex SCF(betaTRCP) [12,13]. Decreased expression of Pdcd4 contributes to tumor development in different ways: Several studies have shown that decreased Pdcd4 expression increases the mobility and invasiveness of tumor cells [8,11,14,15]. In addition, decreased Pdcd4 expression has been shown to impair the cellular DNA-damage response [16–19].

Pdcd4 encodes a highly conserved, predominantly nuclear/cytoplasmic phosphoprotein [20,21] whose subcellular localization is controlled by protein kinase Akt-mediated phosphorylation [21]. Pdcd4 contains two so-called MA-3 domains located in the central and the C-terminal parts of the protein, and an N-terminal RNA-binding domain. Two main activities have been attributed to Pdcd4. Several studies have shown that Pdcd4 modulates the transcription of specific genes by affecting the activities of certain transcription factors, such as c-Jun [22,23], Sp1 [15], Twist1 [24] and p53 [16,19]. In addition, Pdcd4 acts as a translation suppressor. Pdcd4 interacts *via* its MA-3 domains with the eukaryotic translation initiation factor eIF4A and inhibits its RNA helicase activity, which is required for unwinding secondary structures in 5′-untranslated regions (UTRs) of mRNAs [25–31]. It is therefore thought that Pdcd4 suppresses the translation of mRNAs containing 5′ structured UTRs. This has been confirmed using artificial RNAs with stable hairpin structures in their 5′-UTRs [25,26] and by the identification of p53 mRNA as a physiological Pdcd4 target mRNA [18]. Recently, a further mechanism of translational suppression by Pdcd4 has been identified which involves direct RNA-binding of Pdcd4 to target mRNAs [32,33].

Here, we have investigated the influence of Pdcd4 on the expression of protein kinase Hipk2. Hipk2 is a highly conserved protein kinase that

* Corresponding author. Tel.: +49 251 83 33203; fax: +49 251 83 33206.
E-mail address: klempna@uni-muenster.de (K.-H. Klempnauer).
[1] Shared first authorship.

http://dx.doi.org/10.1016/j.bbamcr.2015.03.008
0167-4889/

has been implicated in important biological processes, such as the response to DNA damage [34–36]. We have previously shown that Pdcd4 interferes with the phosphorylation of p53 by Hipk2 [19]. During this work we observed that Pdcd4 also inhibits the expression of Hipk2. We have now explored the effect of Pdcd4 on Hipk2 expression in more detail. We show that Pdcd4 reduces Hipk2 expression by interfering with an auto-regulatory feedback mechanism, by which Hipk2 stimulates the translation of its own mRNA. Our data reveal a novel function of Pdcd4 and provide the first evidence for translational control of Hipk2 expression.

2. Materials and methods

2.1. Cells

Human Hela, HEK293 and HepG2 cells, quail QT6 cells and chicken DF-1 cells were grown in Iscove's modified DMEM supplemented with 10% fetal calf serum. Proteasome inhibitor MG132 and caspase inhibitor z-VAD-FMK were used at 10 and 20 μM, respectively. Cycloheximide was used at a final concentration of 50 μg/ml.

2.2. Expression vectors and transfections

Expression vectors for HA-tagged Hipk2 (pLNCX-HA-Hipk2-wt) and a HA-tagged kinase-dead mutant of Hipk2, carrying a K221A mutation (pLNCX-HA-Hipk2-KD), were obtained from T. Hofmann [37]. Hipk2 deletion constructs Hipk2-ΔN170, Hipk2(1–170) and Hipk2(70–220) have been described [38]. Expression vectors for Flag-tagged Hipk1 and a kinase-dead mutant (Hipk1-K219A) have been described [39] and were subcloned into pCDNA3. The expression vectors for chicken and human Pdcd4 have been described [20,40]. The following expression vectors for mutant versions of human Pdcd4 were used: pCDNA4-hPdcd4-ΔRBD and pCDNA4-hPdcd4-RBM1+2 encode RNA-binding deficient Pdcd4 proteins lacking amino acids 16 to 150 or harboring several amino acid substitutions in the RNA-binding domain, respectively. Both mutants have been described before [32,40]. pCDNA4-hPdcd4-mut4 encodes an eIF4A binding-deficient mutant of human Pdcd4 in which Glu-249, Asp-253, Asp-414, and Asp-418 were changed to Ala [18]. pCDNA4-hPdcd4-RDBstop, pCDNA4-hPdcd4-stop300 and pCDNA4-hPdcd4-stop435 encode human Pdcd4 deleted after amino acids 154, 300 and 435 and were generated by introducing stop codons at the corresponding positions in the coding sequence. Transient transfection of plasmid DNAs into QT6 or Hela cells was performed by calcium phosphate co-precipitation, as described [16].

2.3. CRISPR/Cas9 mediated silencing of Pdcd4

The CRISPR/Cas9 expression vector pSpCas9n(BB)-2A-puro [41], which encodes a mutated "nickase" version of Cas9 and contains a cloning site for a guide RNA, was obtained from Addgene. Guide-RNA target sequences were selected as described [42]. We generated two plasmids targeting the sequences CCTTAGTCGCCTTTTTGCCTTGG (Pdcd4 antisense strand) and GGCGATTCGGTCAGCGACAGTGG (Pdcd4 sense strand) and transfected them simultaneously into HEK293 cells using Metafectene™ (Biontex, Munich, Germany). No off-targets are predicted for this pair of guide-RNAs. Transfectants were enriched by puromycin selection and individual clones were analyzed by western blotting for Pdcd4 expression. Two independent Pdcd4-deficient clones, referred to as K4 and K13, were used for further analysis.

2.4. Northern blotting

Polyadenylated RNA was prepared from transfected cells as described before [16]. Hipk1, Hipk2, GFP and ribosomal protein S17 mRNAs were detected using ^{32}P-radiolabeled probes derived from the corresponding cDNA clones.

2.5. Pulse-labeling with ^{35}S-methionine and immunoprecipitation

Cells transfected with the desired expression vectors were labeled with ^{35}S-methionine (Hartmann Analytics, >800 Ci/mmol) in methionine-free medium at 100 μCi/ml for the desired times. The cells were then lysed in RIPA buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 0.5% NP-40; 0.5% sodium desoxycholate; 0.1% SDS). After incubation on ice for 30 min, lysates were centrifuged at 14,000 ×*g* for 30 min and the supernatant was immunoprecipitated with the appropriate antibodies for 1 to 2 h at 4 °C. Protein-A Sepharose beads were then added and incubated further for 3 h at 4 °C under constant agitation. Immune complexes bound to the beads were collected by centrifugation, washed five times with RIPA buffer, once with PBS and bound proteins were visualized by SDS-PAGE and autoradiography.

2.6. Antibodies

Pdcd4 was detected by a rabbit antiserum raised against the aminoterminus of human Hipk2 [16]. Antibodies against Hipk2 were kindly provided by Lienhard Schmitz. Anti-HA, anti-Flag and anti-Myc antibodies were obtained from commercial suppliers.

3. Results

3.1. Pdcd4 suppresses the translation of Hipk2 mRNA

In a recent study on the effect of Pdcd4 on Hipk2-dependent phosphorylation of p53 we noted that the expression of Hipk2 in cells transfected with a Hipk2 expression vector was reduced when Pdcd4 was co-expressed [19]. To confirm this initial observation we co-transfected expression vectors for Hipk2 and Pdcd4 and analyzed the amounts of both proteins by western blotting (Fig. 1A). Increasing concentrations of Pdcd4 decreased the amount of Hipk2 expressed from a constant amount of Hipk2 expression vector whereas the expression of an unrelated protein, YFP, was not affected by Pdcd4 (bottom panel of Fig. 1A), arguing against an unspecific effect of Pdcd4. Similar co-transfections performed in different cell lines demonstrated that the suppressive effect of Pdcd4 was not cell line specific (Fig. 1B). Furthermore, co-transfection of an expression vector for chicken instead of human Pdcd4 (Fig. 1C) also decreased Hipk2 expression, indicating that the ability to inhibit Hipk2 expression is conserved between human and chicken Pdcd4.

To demonstrate that Pdcd4 also inhibits the expression of endogenous Hipk2 in un-transfected cells we generated Pdcd4-deficient subclones of the HEK293 cell line by CRISPR (clustered regulatory interspaced palindromic repeat)/Cas9-induced genome engineering. Previous work has shown that human codon-optimized Cas9 nuclease can be targeted by a guide-RNA to specific sites in the human genome to induce cleavage at the desired locus [41,43]. We constructed two targeting vectors encoding the "nickase" mutant of human codon-optimized Cas9 and single guide RNAs targeting the human Pdcd4 gene. Independent clones of HEK293 cells transfected simultaneously with both constructs were then selected and tested by western blotting for Pdcd4 expression. We then analyzed the expression of Hipk2 in wild-type HEK293 cells and two of the obtained Pdcd4-deficient subclones. As shown in Fig. 1D both Pdcd4-deficient clones expressed higher levels of Hipk2 than the wild-type cells. This demonstrated that loss of Pdcd4 expression leads to higher Hipk2 expression, consistent with the notion that Pdcd4 suppresses Hipk2 expression also when expressed at the endogenous level.

To find out how Pdcd4 suppresses Hipk2 expression we first investigated whether Pdcd4 decreases the level of Hipk2 mRNA transcribed from the Hipk2 expression vector. However, this was not the case (Fig. 2A). This suggested that Pdcd4 either promotes the degradation of Hipk2 or suppresses its synthesis. To distinguish between these possibilities we first investigated the influence of proteasome (MG132)

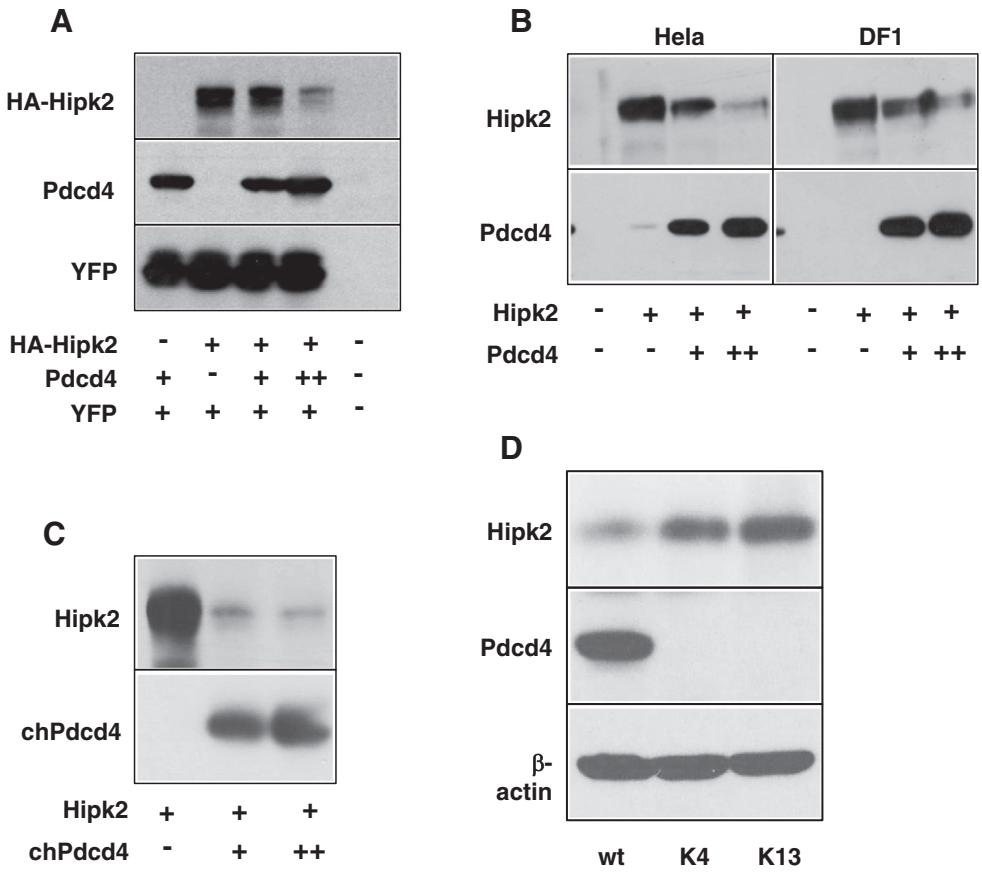

Fig. 1. Inhibition of Hipk2 expression by Pdcd4. A. QT6 fibroblasts were transfected with the indicated combinations of plasmids encoding human Hipk2, YFP and human Pdcd4 together with the β-galactosidase plasmid pCMVβ to monitor transfection efficiencies. 24 h later β-galactosidase-normalized amounts of total protein extracts were analyzed by western blotting with antibodies against the HA-tag, Pdcd4 and GFP. B. Hela cells and chicken DF-1 cells were transfected with expression vector for HA-Hipk2 and human Pdcd4. Cells were analyzed as described in A. C. QT6 cells were transfected with expression vectors for HA-tagged human Hipk2 and chicken Pdcd4 and analyzed as above. D. Western blot analysis of wild-type HEK293 cells (wt) and two Pdcd4-negative HEK293 subclones (K4 and K13) generated by CRISPR/Cas9-mediated genome editing.

and caspase (z-VAD-FMK) inhibitors on the suppression of Hipk2 expression by Pdcd4. If Pdcd4 promotes the degradation of Hipk2 we expected the inhibitory effect of Pdcd4 to be abrogated by these inhibitors. Although the amount of Hipk2 was increased by MG132 the ability of Pdcd4 to suppress Hipk2 expression was not affected by MG132 or the caspase inhibitor (Fig. 2B). It therefore appeared unlikely that Pdcd4 suppresses Hipk2 expression by promoting its degradation. To confirm this we compared the half-life of Hipk2 in the absence and presence of Pdcd4. Cells expressing Hipk2 alone or together with Pdcd4 were treated with cycloheximide to block *de novo* protein synthesis. The cells were then cultivated further in the presence of cycloheximide and the amount of Hipk2 remaining after different times was analyzed by western blotting. Consistent with the previous experiments the amount of Hipk2 was significantly higher in the absence than in the presence of Pdcd4, however, the decrease of Hipk2 over time was very similar in both cases (Fig. 2C). We estimate the half-life of Hipk2 to be approximately 2 h in the presence or absence of Pdcd4. Finally, we performed a metabolic labeling experiment in which cells transfected with Hipk2 or Hipk2 plus Pdcd4 expression vectors were labeled for 20 min with radioactive methionine, harvested immediately and analyzed by immunoprecipitation. Because the labeling period is relatively short compared to the estimated half-life of the protein, the amount of labeled protein is primarily dependent on the rate of its synthesis. The fact that significantly more labeled Hipk2 was synthesized in the absence of Pdcd4 than in its presence directly demonstrated that Pdcd4 suppresses the translation of Hipk2 mRNA.

3.2. Suppression of Hipk2 expression by Pdcd4 requires the N-terminal domain of Pdcd4 but is independent of the ability of Pdcd4 to interact with RNA or eIF4A

Previous work has implicated the ability of Pdcd4 to interact with eIF4A as well as its RNA binding activity in the translational suppression of specific mRNAs [18,25,26,32,33]. To investigate which of these activities, if any, are required for the suppression of Hipk2 expression we examined the inhibitory activity of several Pdcd4 mutants. Fig. 3 shows that a truncated form of Pdcd4 containing only the RNA-binding domain was able to suppress Hipk2 expression whereas the C-terminal part of Pdcd4 without the RNA binding domain was barely active. This demonstrated that the MA-3 domains and the ability of Pdcd4 to interact with the translation initiation factor eIF4A are not involved in the suppression of Hipk2 expression. To find out whether the RNA-binding activity of Pdcd4 is required for suppression of Hipk2 expression we used a Pdcd4 mutant that lacks RNA-binding activity. In this mutant several arginine and lysine residues that are essential for the RNA-binding activity of Pdcd4 have been replaced by alanine [40]. The experiment showed that the RNA-binding defective mutant still inhibited Hipk2 expression (Fig. 3). This indicated that the RNA-binding activity of Pdcd4 is not required for the suppression of Hipk2 expression. Fig. 3 also shows that a Pdcd4 protein carrying mutations in the MA-3 domains that abolish the binding of eIF4A was also able to suppress Hipk2 expression, confirming that the suppressive effect of Pdcd4 is not mediated by interaction with eIF4A. We also performed RNA-immunoprecipitation

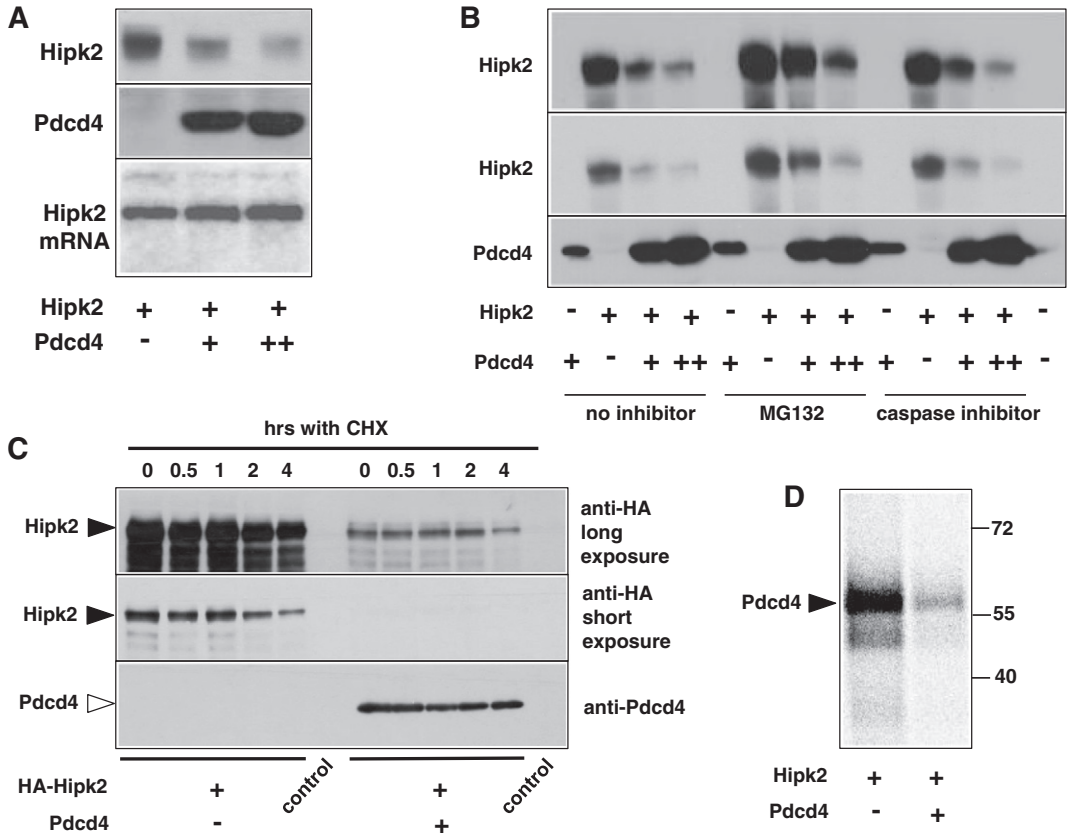

Fig. 2. Pdcd4 suppresses translation of Hipk2 mRNA. A. QT6 cells were transfected with expression vectors for HA-Hipk2 and Pdcd4. Cells were harvested after 24 h and analyzed for protein and RNA expression. Top and middle panel: Aliquots of the cells were analyzed by western blotting with antibodies against the HA-tag and Pdcd4. Bottom panel: Northern blot analysis of RNA isolated from the transfected cells. B. QT6 cells were transfected as indicated at the bottom. 24 h later the cells and treated with 10 μM of the proteasome inhibitor MG132 for 4 h or with the caspase inhibitor z-VAD-FMK (20 μM) for 4 h or incubated without inhibitor. Total cell extracts were then analyzed by western blotting with antibodies against Hipk2 and Pdcd4. The middle panel depicts a shorter exposure of the upper panel to show that the Pdcd4-induced inhibition of Hipk2 is virtually identical in the presence or absence of MG132. C. QT6 cells were transfected with expression vectors for HA-Hipk2 and Pdcd4. 24 h after transfection 50 μg/ml cycloheximide was added to the growth medium and the cells were harvested immediately or after further incubation, as indicated at the top. Total cell extracts were analyzed by western blotting with anti-HA antibodies (Top and middle panels) or Pdcd4 antibodies (bottom panel). The middle panel shows a shorter exposure of the top panel. Untransfected cells are marked as control. D. QT6 cells were transfected as in C. 24 h after transfection the cells were labeled for 20 min with 35S-methionine, lysed and immunoprecipitated with antiserum against the HA-tag, followed by SDS-PAGE and autoradiography. Molecular weight markers are indicated in kilodaltons.

experiments to see if Pdcd4 is associated with Hipk2 mRNA. However, these experiments failed to provide evidence that Pdcd4 is bound to Hipk2 mRNA (data not shown), suggesting that Pdcd4 suppresses translation of Hipk2 mRNA by a novel mechanism.

3.3. Hipk2 stimulates translation of its own mRNA

During this work we observed that a full-length kinase-dead construct of Hipk2 containing only a single amino acid replacement (K221A) was expressed at significantly lower levels than the corresponding wild-type construct, although the same amounts of expression vector were transfected. In addition, the wild-type kinase had a higher apparent molecular weight than the kinase-dead mutant (Fig. 4A). Work from several groups has shown that Hipk2 is able to phosphorylate itself to give rise to a form with slower electrophoretic mobility in SDS-polyacrylamide gels [37,44–47]. Northern blot analysis of cells transfected with wild-type or kinase-dead Hipk2 expression vectors showed that the difference in the expression of both proteins was not due to differential transcription (Fig. 4B). Because phosphorylation might affect the stability of the protein we wondered whether the different expression levels of wild-type and kinase-dead Hipk2 were due to a difference of the half-life between the wild-type and the mutant protein. To address this possibility, we compared the expression levels of wild-type and mutant Hipk2 in the absence and presence of the proteasome inhibitor MG132. We reasoned that if the mutant protein was less stable than the wild-type protein, inhibition of proteasomal degradation would cause the expression levels of both proteins to become more similar or identical. Fig. 4C shows that both proteins were still expressed at very different levels even when degradation was inhibited. In addition, we measured the half-life of wild-type and kinase-dead Hipk2 by treating cells transfected with expression vectors for both forms of Hipk2 with cycloheximide and following the degradation of both proteins over several hours (Fig. 4D). Both proteins showed a very similar decrease over time. Finally, we also performed a pulse-labeling experiment of cells transfected with equal amounts of expression vectors for wild-type and kinase-dead Hipk2. The cells were labeled with radioactive methionine for 30 min, harvested immediately and analyzed by immunoprecipitation with antibodies against the HA-tag (Fig. 4E). Since the labeling period was short compared to the estimated half-life of the protein the difference of the amounts of labeled proteins reflects primarily the difference in the rate of their synthesis. Taken together, these experiments showed that the differential expression of wild-type and kinase-dead Hipk2 was not due to differential stability of both proteins but to a higher rate of synthesis of the wild-type protein. Since the RNA amounts for both proteins were not different, we concluded that the mRNA for wild-type Hipk2 is translated more efficiently than the RNA for the kinase-dead mutant.

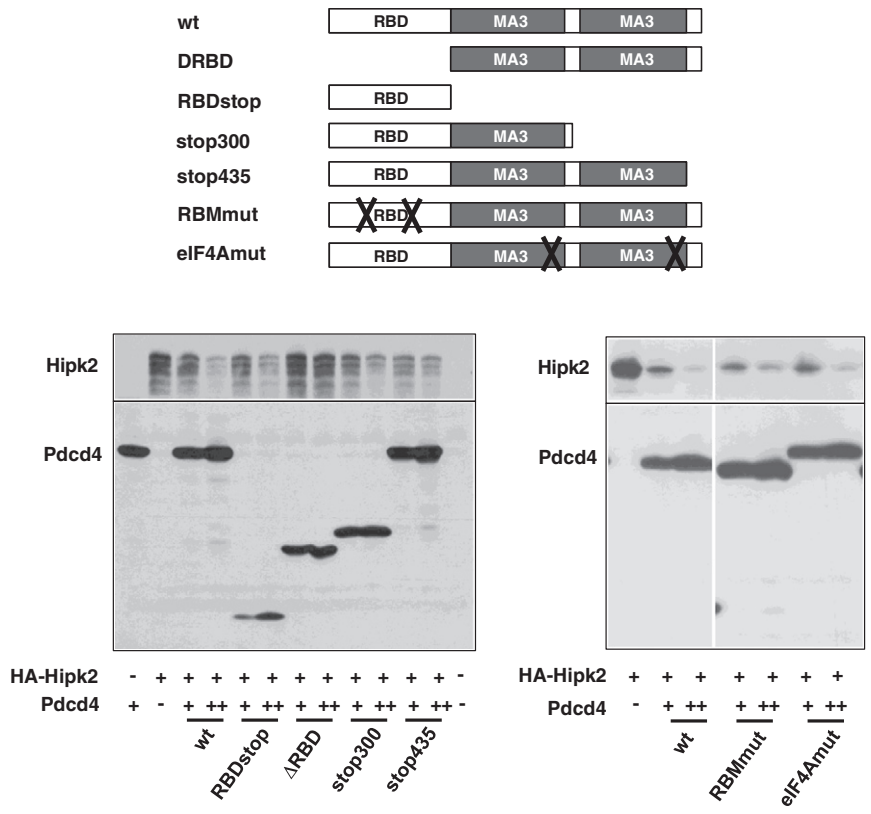

Fig. 3. Functional domains of Pdcd4 required for suppression of Hipk2 expression by Pdcd4. The top shows a schematic illustration of different Pdcd4 mutants. The RNA-binding and MA-3 domains are highlighted. Point mutations are indicated by crosses. Bottom panels: QT6 cells were transfected with expression vectors for HA-tagged Hipk2 and wild-type or mutant Pdcd4, as indicated. 24 h later total protein extracts were analyzed by western blotting using antibodies against Pdcd4 and the HA-tag.

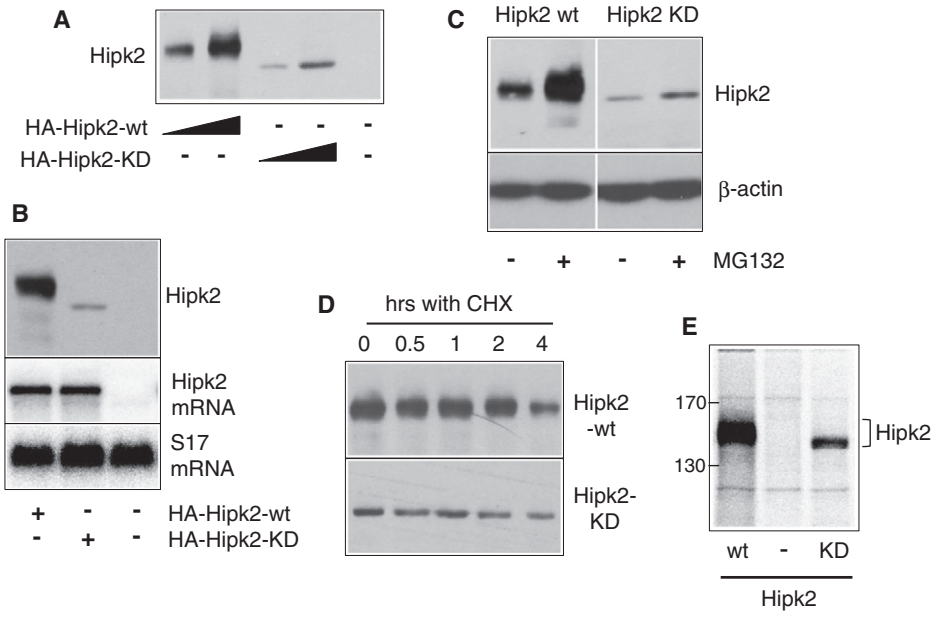

Fig. 4. Translation of a kinase-dead Hipk2 mutant is impaired compared to translation of wild-type Hipk2. A. QT6 cells were transfected with identical amounts of expression vectors for HA-tagged wild-type and a kinase-dead mutant of Hipk2, as indicated. Total cell extracts were analyzed after 24 h by western blotting using antibodies against the HA-tag. B. QT6 cells were transfected with identical amounts of expression vectors for HA-tagged wild-type or kinase-dead Hipk2. Cells were analyzed 24 h after transfection for Hipk2 expression (top panel). Polyadenylated RNA isolated from aliquots of the transfected cells was analyzed by northern blotting with Hipk2-specific (middle panel) and ribosomal S17-specific probes (bottom panel). C. QT6 cells were transfected with identical amounts of expression vectors for HA-tagged wild-type or kinase-dead Hipk2. After 24 h the cells were treated for 4 h with or without 10 μM MG132 before they were harvested. Total cell extracts were then analyzed by western blotting with antibodies against the HA-tag. D. QT6 cells were transfected with expression vectors for HA-tagged wild-type or the kinase-dead Hipk2. 24 h after transfection 50 μg/ml cycloheximide was added to the growth medium and the cells were harvested at the times indicated at the top. Total cell extracts were analyzed by western blotting with anti-HA antibodies. E. QT6 cells were transfected with equal amounts of expression vectors for HA-tagged wild-type and a kinase-dead mutant of Hipk2 or were not transfected, as indicated below. 24 h after transfection the cells were labeled for 30 min with 35S-methionine, lysed and immunoprecipitated with antiserum against the HA-tag, followed by SDS-PAGE and autoradiography. Molecular weight markers are indicated in kilodaltons.

In considering possible explanations for the different translation efficiencies of both RNAs we thought it unlikely that replacement of one amino acid and the corresponding difference in the nucleotide sequence between both RNAs could explain the difference in translation efficiency. We therefore considered the possibility that wild-type Hipk2, but not the kinase-dead mutant, stimulates translation of its own mRNA by a feed-back mechanism. If such a mechanism existed one might expect that kinase-active Hipk2 would stimulate the translation of mRNA encoding kinase-dead Hipk2 by a "trans-stimulatory" mechanism. To test this idea we co-expressed kinase-dead Hipk2 with or without active but truncated forms of Hipk2 and analyzed protein as well as mRNA levels (Fig. 5A). This experiment showed that the presence of the active Hipk2/1-541 or Hipk2/1-860 caused a significant increase of the expression of the kinase-dead Hipk2 although the amount of the mRNA encoding the kinase-dead Hipk2 remained the same. This demonstrated that active forms of Hipk2 are able to stimulate the translation of Hipk2 RNA. When the kinase-dead instead of the kinase-active form of truncated Hipk2/1-860 was used, no stimulation of the expression of the full-length kinase-dead Hipk2 occurred (Fig. 5B), demonstrating that the kinase activity plays a crucial role in increasing the translation of Hipk2 RNA. We also observed a large difference in the expression levels of the kinase-active and kinase-dead versions of the truncated Hipk2/1-860 construct, most likely because the kinase-active construct stimulates its own expression. Taken together, these observations indicated that Hipk2 stimulates the translation of its own mRNA by a mechanism that depends on its kinase activity.

We wondered whether the stimulation of the translation of Hipk2 mRNA by Hipk2 is a peculiarity of the QT6 fibroblast line used in most of the experiments described so far, or whether it also occurs in other cells, particularly in human cells. We therefore transfected Hela cells with equivalent amounts of expression vectors for wild-type and kinase-dead Hipk2 and analyzed the resulting amounts of Hipk2 by western blotting. Fig. 5C shows that the expression of the wild-type kinase was significantly higher than the expression of the kinase-dead mutant, suggesting that wild-type Hipk2 stimulates its expression also in human cells. To further support this conclusion we transfected Hela cells with expression vectors for Myc-tagged full-length kinase-dead Hipk2 and the HA-tagged truncated form of the active kinase (Fig. 5D). This experiment showed that the amount of the kinase-dead mutant was strongly increased by the active kinase, confirming that Hipk2 stimulates translation of its own mRNA also in human cells.

3.4. Pdcd4 interferes with the auto-stimulation of Hipk2 translation

Since our data showed that Pdcd4 suppresses the translation of Hipk2 mRNA we were interested to know whether or not this inhibitory function of Pdcd4 is related to the auto-stimulation of Hipk2 translation. If the inhibitory activity of Pdcd4 was unrelated to the auto-stimulation of Hipk2 expression, Pdcd4 is expected to suppress the expression of wild-type and kinase-dead Hipk2 to similar extent. If, on the other hand, Pdcd4 specifically interferes with the auto-stimulation of Hipk2 expression, Pdcd4 should have a much stronger effect on wild-type than on kinase-dead Hipk2 expression. Fig. 6A shows that the expression of kinase-dead Hipk2 was only slightly affected by Pdcd4 while wild-type Hipk2 was strongly inhibited under the same conditions. This indicated that Pdcd4 does not suppress translation of Hipk2 mRNA *per se* but that it specifically interferes with the auto-stimulatory function of Hipk2, as depicted schematically in Fig. 6B.

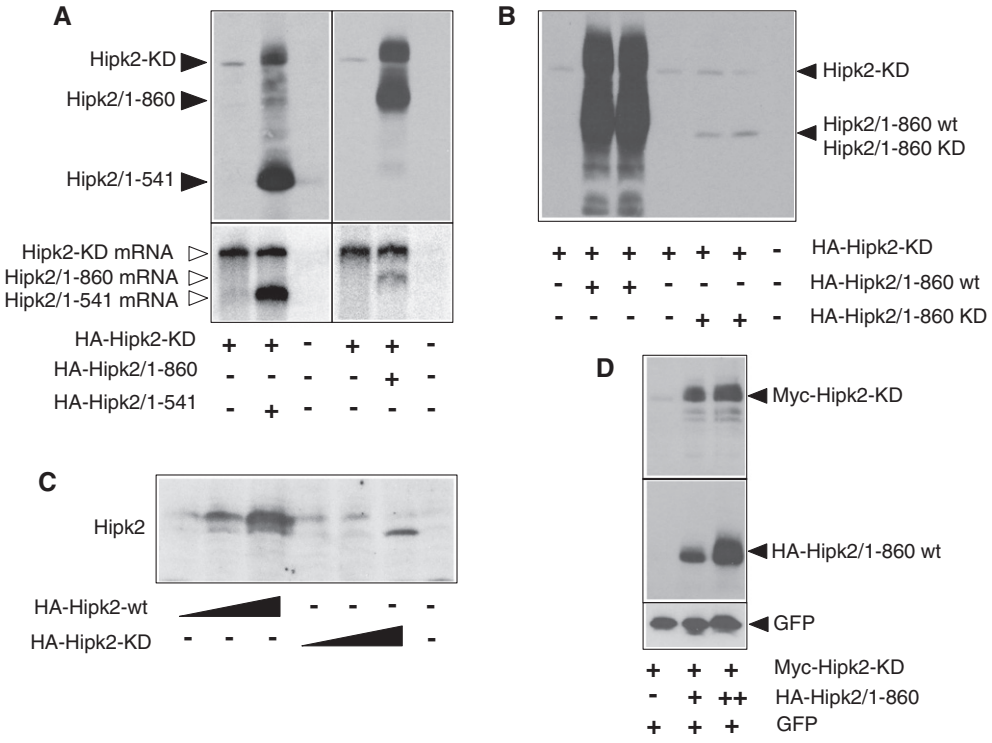

Fig. 5. Hipk2 stimulates the translation of its own mRNA. A. Cells were transfected with expression vectors for full-length kinase-dead and two different C-terminally truncated kinase-active Hipk2 constructs, as indicated. Cells were analyzed 24 h after transfection for Hipk2 expression (top panel). Polyadenylated RNA isolated from aliquots of the transfected cells was analyzed by northern blotting with Hipk2-specific probe (bottom panel). B. QT6 cells were transfected with expression vectors for kinase-dead full-length HA-Hipk2 and active or kinase-dead versions of C-terminally truncated HA-Hipk2, as indicated at the bottom. Total cell extracts were analyzed 24 h later by western blotting with antibodies against the HA-tag. C. Hela cells were transfected with equivalent amounts of expression vectors for HA-tagged wild-type and a kinase-dead mutant of Hipk2, as indicated. Total cell extracts were analyzed after 24 h by western blotting with antibodies against the HA-tag. D. Hela cells were transfected with expression vectors for Myc-tagged full-length kinase-dead Hipk2, HA-tagged truncated kinase-active Hipk2 and GFP, as indicated at the bottom. Total cell extracts were analyzed 24 h later by western blotting with antibodies against the Myc- and HA-tag and GFP (top, middle and bottom panels, respectively).

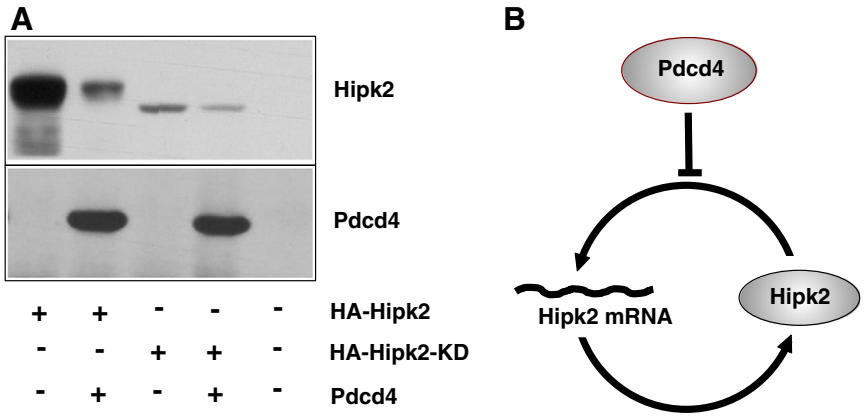

Fig. 6. Pdcd4 disrupts the auto-stimulatory activity of Hipk2. A. QT6 cells were transfected with expression vectors for Pdcd4 and the indicated wild-type and kinase-dead forms of Hipk2. Total cell extracts were analyzed 24 h later by western blotting with antibodies against Pdcd4 and the HA-tag. B. Model for the suppression of Hipk2 expression by Pdcd4.

3.5. Hipk2 and Pdcd4 cooperate to modulate the expression of Hipk1

Finally, we wondered if the cooperation of Hipk2 and Pdcd4 would affect only the expression of Hipk2 or if heterologous RNAs might also be regulated in similar manner by both proteins. As a first step, we focused on the expression of protein kinase Hipk1 as a model and co-transfected different combinations of expression vectors for wild-type or kinase-dead Hipk1 and Pdcd4, followed by western blot analysis to determine the expression levels of Hipk1 (Fig. 7A). Wild-type Hipk1 was expressed at significantly higher levels than the kinase-dead form

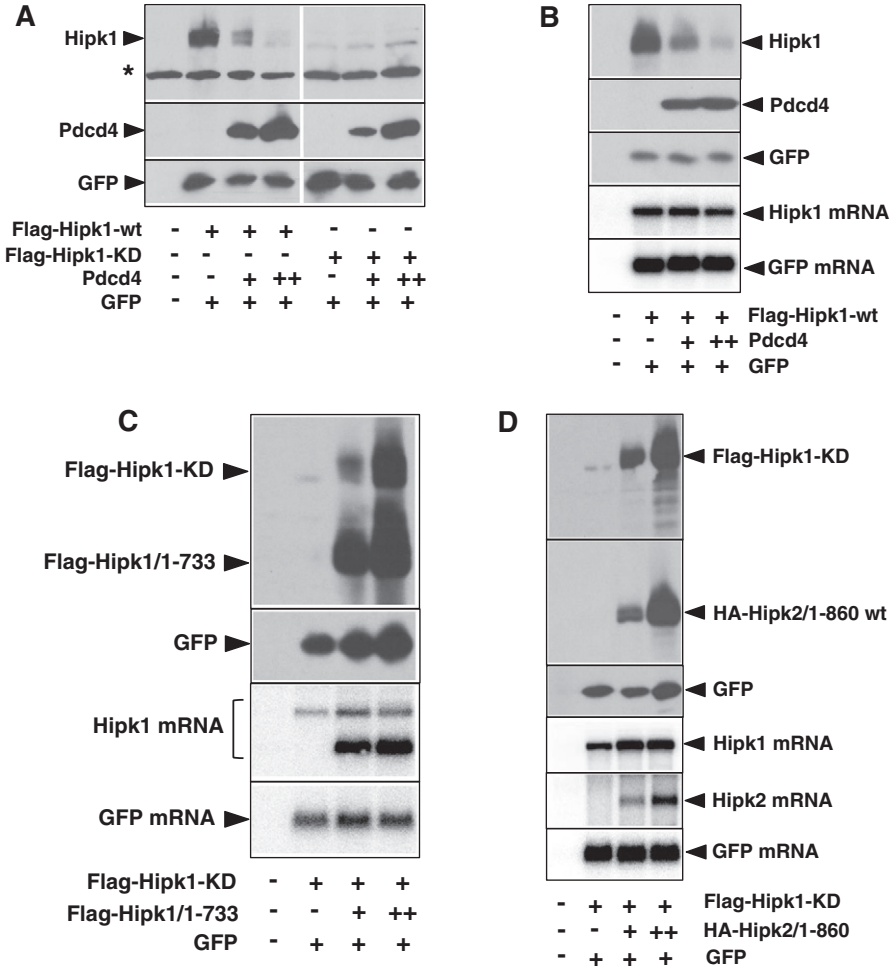

Fig. 7. Influence of Pdcd4 and Hipk2 on Hipk1 expression. A. QT6 cells were transfected with the indicated combinations of expression vectors for full-length wild-type and kinase-dead Flag-tagged Hipk1, Pdcd4 and GFP. Total cell extracts were analyzed 24 h later by western blotting with antibodies against Pdcd4, GFP and the Flag-tag. B–D. QT6 cells were transfected with the indicated expression vectors and analyzed by western blotting as in A (upper parts of each panel). In addition, polyadenylated RNA was isolated from aliquots of the transfected cells and analyzed by northern blotting (lower parts of each panel).

of Hipk1, suggesting that Hipk1 might also be regulated by a similar feed-back mechanism as Hipk2. Furthermore, Pdcd4 suppressed the expression of the active form of Hipk1 but not that of the kinase-dead mutant. As in the case of Hipk2, the decrease of Hipk1 expression by Pdcd4 was not accompanied by a decrease of the Hipk1 mRNA levels (Fig. 7B). The difference in the expression levels of wild-type and kinase-dead Hipk1 suggested that Hipk1, like Hipk2, might also regulate its translation by an autoregulatory mechanism. To test this, we co-transfected expression vectors for full-length kinase-dead Hipk1 and an active but C-terminally truncated form of Hipk1. Fig. 7C shows that the amount of the kinase-dead Hipk1 was strongly increased by co-expression of active Hipk1 without concurrent increase of the mRNA level of kinase-dead Hipk1. This suggested that Hipk1, like Hipk2 stimulates translation of its own mRNA. Finally, we were interested to know if the kinases stimulate only the translation of their own RNAs or if they can "cross-stimulate" each other. As shown in Fig. 7D, co-expression with an active form of Hipk2 strongly increased the expression of the kinase-dead form of Hipk1, demonstrating that Hipk2 also stimulates the translation of Hipk1 RNA.

4. Discussion

Our work reveals an unexpected and novel link between Pdcd4 and the expression of the protein kinase Hipk2 and thereby provides new insight into the role of Pdcd4 as a translation suppressor as well as into the control of Hipk2 expression. Hipk2 is an evolutionary conserved protein kinase which plays important roles in different biological processes ranging from the response to DNA damage and oxidative stress to hypoxia signaling and the control of apoptosis, proliferation and differentiation [48,49]. This multitude of important functions requires precise control of Hipk2 activity to prevent dysregulation of these processes and the development of diseases, such as cancer. Unlike many other protein kinases Hipk2 appears not to require activating events after its synthesis, such as the phosphorylation by an "upstream activating kinase". Rather, Hipk2 activates itself by autophosphorylation during its synthesis and appears to be regulated by the modulation of its stability [37,44–47]. Recent studies have revealed a complex network of Hipk2 interacting proteins that control the ubiquitin-dependent degradation of Hipk2. The function of this regulatory network has been explored in detail in the response to DNA-damage and hypoxia [48–51]. Under normal conditions the amount of Hipk2 is low due to its interaction with several E3 ubiquitin ligases, including Siah1 and 2, WSB-1 and Fbx3, which mediate the ubiquitination and subsequent degradation of Hipk2. Following stress (*e.g.* DNA damage) Hipk2 is released from the ubiquitin ligases, which leads to its stabilization and increased phosphorylation of its downstream targets.

The work presented here adds a completely new aspect to the picture of Hipk2 regulation by providing the first evidence that the expression of Hipk2 is also controlled on the level of translation of Hipk2 mRNA. Our work demonstrates that Hipk2 controls an autoregulatory feed-back loop by which it stimulates its own synthesis. The fact that the kinase-dead Hipk2 mutant was not able to increase its translation, indicates that this feed-back stimulation requires the kinase activity of Hipk2 and suggests that the phosphorylation of an unknown downstream target of Hipk2 is responsible for the increased translation of Hipk2 mRNA. Our initial analysis of the expression of Hipk1 has shown that translation of Hipk1 is regulated by a similar feed-back mechanism as that of Hipk2. Moreover, we have shown that the expression of kinase-dead Hipk1 is also stimulated by Hipk2. This raises the interesting possibility that Hipk2 stimulates not only the translation of its own but also of other mRNAs, as exemplified by Hipk1 mRNA. It will therefore be interesting to investigate the mechanistic basis of this new function of Hipk2 and to search for mRNAs whose translation is affected by Hipk2.

A second conclusion of our work is that Pdcd4 affects Hipk2 expression by interfering with the autostimulation of Hipk2 mRNA translation. This indicates that the Hipk2-dependent feedback loop is not constitutively active, but that it is controlled by Pdcd4 and perhaps other factors. Pdcd4 itself is also regulated in a complex manner, including phosphorylation of Pdcd4 by S6- and Akt-kinases and silencing of Pdcd4 expression by microRNA-21 [9–11]. Thus, in addition to the control of Hipk2 stability there appears to be an additional complex layer of regulation of Hipk2 expression that acts on the level of translation.

Our work also provides new insight into the function of Pdcd4 as a translation suppressor. Because Pdcd4 interacts with the translation initiation factor eIF4A and inhibits its helicase activity, it was initially proposed that Pdcd4 suppresses the translation of mRNAs containing structured 5′-UTRs in an eIF4A-dependent manner [25,26]. It now appears that the role of Pdcd4 as translation suppressor is more complex. Our data show that Pdcd4 is involved in translational suppression of Hipk2 mRNA by a mechanism that is not dependent on the Pdcd4-eIF4A interaction. Our work shows that the suppression of Hipk2 expression is dependent on the N-terminal domain of Pdcd4. This part of Pdcd4 is involved in protein–protein-interactions, for example with Daxx, PRMT5 and the poly(A)-binding protein [19,52,53], but also functions as an RNA-binding domain [32,41]. We have recently shown that Pdcd4 suppresses the translation of c-*myb* and A-*myb* mRNAs by directly binding to the RNA [32,33]. Direct Pdcd4-RNA binding seems not to be involved in the case of Hipk2 mRNA because a RNA-binding defective Pdcd4 mutant was still able to suppress Hipk2 translation. How Pdcd4 acts mechanistically to inhibit the Hipk2-dependent feed-back stimulation of its own synthesis is not clear at present. A possible scenario is that Pdcd4 modifies the expression or activity of a downstream effector, *e.g.* a RNA-binding protein or regulatory RNA that interacts with Hipk2 RNA to affect its translation. In any case, our work adds a novel aspect to the current picture of mechanisms by which Pdcd4 affects the translation of cellular mRNAs.

As Hipk2 plays important roles in numerous important biological processes, Pdcd4 is likely to exert pleiotropic effects on these processes and thereby influences cellular homeostasis by multiple mechanisms. Hipk2 has recently been shown to phosphorylate the cyclin-dependent kinase inhibitor p27(Kip1) at Ser10, leading to the stabilization of p27(Kip1) and increased cell motility [54]. A decrease of Pdcd4, as observed in many tumor cells, might therefore stimulate cell motility *via* increased expression of Hipk2 and subsequent phosphorylation of p27(Kip1). This is consistent with several studies that have shown that siRNA-mediated knock-down of Pdcd4 leads to increased cell motility [8,11,14,15]. Although Hipk2 is considered to be itself a tumor suppressor [36] there is also evidence for a pro-oncogenic role of Hipk2 in certain tumors, such as brain tumors and cervical carcinomas [55–57], where Hipk2 is frequently overexpressed. Pdcd4 is down-regulated in invasive cervical cancer [58] suggesting that it may counteract Hipk2 in this type of cancer. It is also possible that the effect of Pdcd4 on Hipk2 expression is not directly related to Pdcd4's role as a tumor suppressor. As pointed out, Hipk2 plays a prominent role in the DNA damage response by phosphorylating p53 at Ser46 [37,44]. We have previously reported that Pdcd4 suppresses the phosphorylation of p53 at Ser46 as well as the translation of p53 mRNA in cells growing under normal conditions [18,19]. That Pdcd4 also suppresses Hipk2 expression accords with these observations and supports a model in which Pdcd4 plays a role as a dampening factor that ensures a low level of activity of the Hipk2-p53 axis in unstressed cells. In any case, the identification of the Hipk2-dependent auto-stimulation and the disruption of this mechanism by Pdcd4 open up new perspectives for future studies on the function of Pdcd4 and Hipk2.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

We thank B. Berkenfeld and P. Pieloch for their technical assistance, T. Hofmann and L. Schmitz for Hipk2 expression vectors and antibodies and O. Gabrielsen for Hipk1 expression vectors. This work was supported by the Deutsche Krebshilfe (109269), the Wilhelm-Sander-Stiftung (2008.058.1) and the Deutsche Forschungsgemeinschaft (KL461/15-1). A.B. was supported by the Graduate School of Chemistry (GSC-MS) at the University of Münster.

References

[1] K. Shibahara, M. Asano, Y. Ishida, T. Aoki, T. Koike, T. Honjo, Isolation of a novel mouse gene MA-3 that is induced upon programmed cell death, Gene 166 (1995) 297–301.
[2] J.L. Cmarik, H. Min, G. Hegamyer, S. Zhan, M. Kulesz-Martin, H. Yoshinaga, S. Matsuhashi, N.H. Colburn, Differentially expressed protein Pdcd4 inhibits tumor promoter-induced neoplastic transformation, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 14037–14042.
[3] A.P. Jansen, C.E. Camalier, N.H. Colburn, Epidermal expression of the translation inhibitor programmed cell death 4 suppresses tumorigenesis, Cancer Res. 65 (2005) 6034–6041.
[4] Y. Chen, T. Knosel, G. Kristiansen, A. Pietas, M.E. Garber, S. Matsuhashi, I. Ozaki, I. Petersen, Loss of PDCD4 expression in human lung cancer correlates with tumour progression and prognosis, J. Pathol. 200 (2003) 640–646.
[5] O. Afonja, D. Juste, S. Das, S. Matsuhashi, H.H. Samuels, Induction of PDCD4 tumor suppressor gene expression by RAR agonists, antiestrogen and HER-2/neu antagonist in breast cancer cells. Evidence for a role in apoptosis, Oncogene 23 (2004) 8135–8145.
[6] H. Zhang, I. Ozaki, T. Mizuta, H. Hamajima, T. Yasutake, Y. Eguchi, H. Ideguchi, K. Yamamoto, S. Matsuhashi, Involvement of programmed cell death 4 in transforming growth factor-beta1-induced apoptosis in human hepatocellular carcinoma, Oncogene 25 (2006) 6101–6112.
[7] G. Mudduluru, F. Medved, R. Grobholz, C. Jost, A. Gruber, J.H. Leupold, S. Post, A. Jansen, N.H. Colburn, H. Allgayer, Loss of programmed cell death 4 expression marks adenoma–carcinoma transition, correlates inversely with phosphorylated protein kinase B, and is an independent prognostic factor in resected colorectal cancer, Cancer 110 (2007) 1697–1707.
[8] Q. Wang, Z. Sun, H.-S. Yang, Downregulation of tumour suppressor Pdcd4 promotes invasion and activates both β-catenin/TCF and AP-1-dependent transcription in colon carcinoma cells, Oncogene 27 (2008) 1527–1535.
[9] L.B. Frankel, N.R. Christoffersen, A. Jacobsen, M. Lindow, A. Krogh, A.H. Lund, Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells, J. Biol. Chem. 283 (2008) 1026–1033.
[10] Z. Lu, M. Liu, V. Stribinskis, C.M. Klinge, K.S. Ramos, N.H. Colburn, MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene, Oncogene 27 (2008) 4373–4379.
[11] I.A. Asangani, S.A. Rasheed, D.A. Nikolova, J.H. Leupold, N.H. Colburn, S. Post, H. Allgayer, MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer, Oncogene 27 (2008) 2128–2136.
[12] N.V. Dorello, A. Peschiaroli, D. Guardavaccaro, N.H. Colburn, N.E. Sherman, M. Pagano, S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth, Science 314 (2006) 467–471.
[13] T. Schmid, A.P. Jansen, A.R. Baker, G. Hegamyer, J.P. Hagan, N.H. Colburn, Translation inhibitor Pdcd4 is targeted for degradation during tumor promotion, Cancer Res. 68 (2008) 1254–1260.
[14] H.S. Yang, C.P. Matthews, T. Clair, Q. Wang, A.R. Baker, C.C. Li, T.H. Tan, N.H. Colburn, Tumorigenesis suppressor Pdcd4 down-regulates mitogen-activated protein kinase kinase kinase kinase 1 expression to suppress colon carcinoma cell invasion, Mol. Cell. Biol. 26 (2006) 1297–1306.
[15] J.H. Leupold, H.S. Yang, N.H. Colburn, I. Asangani, S. Post, H. Allgayer, Tumor suppressor Pdcd4 inhibits invasion/intravasation and regulates urokinase receptor (u-PAR) gene expression via Sp-transcription factors, Oncogene 26 (2007) 4550–4562.
[16] N. Bitomsky, N. Wethkamp, R. Marikkannu, K.-H. Klempnauer, siRNA-mediated knock-down of Pdcd4 expression causes up-regulation of p21(Waf1/Cip1) expression, Oncogene 27 (2008) 4820–4829.
[17] P. Singh, R. Marikkannu, N. Bitomsky, K.-H. Klempnauer, Disruption of the Pdcd4 tumor suppressor gene in chicken DT40 cells reveals its role in the DNA-damage response, Oncogene 28 (2009) 3758–3764.
[18] L. Wedeken, P. Singh, K.-H. Klempnauer, Tumor suppressor protein Pdcd4 inhibits translation of p53 mRNA, J. Biol. Chem. 286 (2011) 42855–42862.
[19] N. Kumar, N. Wethkamp, L.C. Waters, M.D. Carr, K.-H. Klempnauer, Tumor suppressor protein Pdcd4 interacts with Daxx and modulates the stability of Daxx and the Hipk2-dependent phosphorylation of p53 at serine 46, Oncogenesis 2 (2013) e37.
[20] M. Böhm, K. Sawicka, J.P. Siebrasse, A. Brehmer-Fastnacht, R. Peters, K.-H. Klempnauer, The transformation suppressor protein Pdcd4 shuttles between nucleus and cytoplasm and binds RNA, Oncogene 22 (2003) 4905–4910.
[21] A. Palamarchuk, A. Efanov, V. Maximov, R.I. Aqeilan, C.M. Croce, Y. Pekarsky, Akt phosphorylates and regulates Pdcd4 tumor suppressor protein, Cancer Res. 65 (2005) 11282–11286.
[22] H.S. Yang, J.L. Knies, C. Stark, N.H. Colburn, Pdcd4 suppresses tumor phenotype in JB6 cells by inhibiting AP-1 transactivation, Oncogene 22 (2003) 3712–3720.
[23] N. Bitomsky, M. Böhm, K.-H. Klempnauer, Transformation suppressor protein Pdcd4 interferes with JNK-mediated phosphorylation of c-Jun and recruitment of the coactivator p300 by c-Jun, Oncogene 23 (2004) 7484–7493.
[24] M. Shiota, H. Izumi, A. Tanimoto, M. Takahashi, N. Miyamoto, E. Kashiwagi, A. Kidani, G. Hirano, D. Masubucki, Y. Fukunaka, Y. Yasuniwa, S. Naito, S. Nishizawa, Y. Sasaguri, K. Kohno, Programmed cell death protein 4 down-regulates Y-box binding protein-1 expression via a direct interaction with Twist1 to suppress cancer cell growth, Cancer Res. 69 (2009) 3148–3156.
[25] H.S. Yang, A.P. Jansen, A.A. Komar, X. Zheng, W.C. Merrick, S. Costes, S.J. Lockett, N. Sonenberg, N.H. Colburn, The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation, Mol. Cell. Biol. 23 (2003) 26–37.
[26] H.S. Yang, M.H. Cho, H. Zacowicz, G. Hegamyer, N. Sonenberg, N.H. Colburn, A novel function of the MA-3 domains in transformation and translation suppressor Pdcd4 is essential for its binding to eukaryotic translation initiation factor 4A, Mol. Cell. Biol. 24 (2004) 3894–3906.
[27] N. LaRonde-LeBlanc, A.N. Santhanam, A.R. Baker, A. Wlodawer, N.H. Colburn, Structural basis for inhibition of translation by the tumor suppressor Pdcd4, Mol. Cell. Biol. 27 (2007) 147–156.
[28] L.C. Waters, V. Veverka, M. Böhm, T. Schmedt, P.T. Choong, F.W. Muskett, K.-H. Klempnauer, M.D. Carr, Structure of the C-terminal MA-3 domain of the tumour suppressor protein Pdcd4 and characterization of its interaction with eIF4A, Oncogene 26 (2007) 4941–4950.
[29] C. Suzuki, R.G. Garces, K.A. Edmonds, S. Hiller, S.G. Hyberts, A. Marintchev, G. Wagner, PDCD4 inhibits translation initiation by binding to eIF4A using both its MA3 domains, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 3274–3279.
[30] J.H. Chang, Y.H. Cho, S.Y. Sohn, J.M. Choi, A. Kim, Y.C. Kim, S.K. Jang, Y. Cho, Crystal structure of the eIF4A-PDCD4 complex, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 3148–3153.
[31] P.G. Loh, H.S. Yang, M.A. Walsh, Q. Wang, X. Wang, Z. Cheng, D. Liu, H. Song, Structural basis for translational inhibition by the tumour suppressor Pdcd4, EMBO J. 28 (2009) 274–285.
[32] P. Singh, L. Wedeken, L.C. Waters, M.D. Carr, K.-H. Klempnauer, Pdcd4 directly binds the coding region of c-myb mRNA and suppresses its translation, Oncogene 30 (2011) 4864–4873.
[33] A. Biyanee, J. Ohnheiser, P. Singh, K.-H. Klempnauer, A novel mechanism for the control of translation of specific mRNAs by tumor suppressor protein Pdcd4: inhibition of translation elongation, Oncogene 34 (2015) http://dx.doi.org/10.1038/onc.2014.83 (Epub ahead of print).
[34] M.A. Calzado, F. Renner, A. Roscic, M.L. Schmitz, Hipk2: a versatile switchboard regulating the transcription machinery and cell death, Cell Cycle 6 (2007) 139–143.
[35] C. Rinaldo, A. Prodosmo, F. Siepi, S. Soddu, Hipk2: a multitalented partner for transcription factors in DNA damage response and development, Biochem. Cell Biol. 85 (2007) 411–418.
[36] T.G. Hofmann, C. Glas, N. Bitomsky, Hipk2: a tumor suppressor that controls DNA damage-induced cell fate and cytokinesis, Bioessays 35 (2013) 55–64.
[37] T.G. Hofmann, A. Möller, H. Sirma, H. Zentgraf, Y. Taya, W. Dröge, H. Will, M.L. Schmitz, Regulation of p53 activity by its interaction with homeo-domain-interacting protein kinase-2, Nat. Cell Biol. 4 (2002) 1–10.
[38] S. Steinmann, A. Coulibaly, J. Ohnheiser, A. Jakobs, K.-H. Klempnauer, Interaction and cooperation of the CCAAT-box enhancer binding protein beta (C/EBPβ) with the homeo-domain interacting protein kinase 2 (Hipk2), J. Biol. Chem. 88 (2013) 22257–22269.
[39] V. Matre, O. Nordgard, A.H. Alm-Kristiansen, M. Ledsaak, O.S. Gabrielsen, HIPK1 interacts with c-Myb and modulates its activity through phosphorylation, Biochem. Biophys. Res. Commun. 388 (2009) 150–154.
[40] L. Wedeken, J. Ohnheiser, B. Hirschi, N. Wethkamp, K.-H. Klempnauer, Association of tumor suppressor protein Pdcd4 with ribosomes is mediated by protein–protein and protein–RNA interactions, Genes Cancer 1 (2010) 293–301.
[41] L. Cong, F.A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P.D. Hsu, X. Wu, W. Jiang, L.A. Marraffini, F. Zhang, Multiplex genome engineering using CRISPR/Cas systems, Science 339 (2013) 819–823.
[42] F.A. Ran, P.D. Hsu, J. Wright, V. Agarwala, D.A. Scott, F. Zhang, Genome editing using the CRISPR-Cas9 system, Nat. Protoc. 8 (2013) 2281–2308.
[43] P. Mali, L. Yang, K.M. Esvelt, J. Aach, M. Guell, J.E. DiCarlo, J.E. Norville, G.M. Church, RNA-guided human genome engineering via Cas9, Science 339 (2013) 823–826.
[44] G. D'Orazi, B. Cecchinelli, T. Bruno, I. Manni, Y. Higashimoto, S. Saito, M. Gostissa, S. Coen, A. Marchetti, G. Del Sal, G. Piaggio, M. Fanciulli, E. Appella, S. Soddu, Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis, Nat. Cell Biol. 4 (2002) 11–19.
[45] V.V. Saul, L. de la Vega, M. Milanovic, M. Krüger, T. Braun, K. Fritz-Wolf, K. Becker, M.L. Schmitz, HIPK2 kinase activity depends on cis-autophosphorylation of its activation loop, J. Mol. Cell Biol. 5 (2013) 27–38.
[46] F. Siepi, V. Gatti, S. Camerini, M. Crescenzi, S. Soddu, HIPK2 catalytic activity and subcellular localization are regulated by activation-loop Y345 autophosphorylation, Biochim. Biophys. Acta 1833 (2013) 1443–1453.
[47] N. Bitomsky, E. Conrad, C. Moritz, T. Polonio-Vallon, D. Sombroek, K. Schultheiss, C. Glas, V. Greiner, C. Herbel, F. Mantovani, G. del Sal, F. Peri, T.G. Hofmann, Autophosphorylation and Pin1 binding coordinate DNA damage-induced HIPK2 activation and cell death, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) E4203–E4212.
[48] M.A. Calzado, L. de la Vega, A. Moller, D.D. Bowtell, M.L. Schmitz, An inducible autoregulatory loop between HIPK2 and Siah2 at the apex of the hypoxic response, Nat. Cell Biol. 11 (2009) 85–91.

[49] C. Rinaldo, A. Prodosmo, F. Mancini, S. Iacovelli, A. Sacchi, F. Moretti, S. Soddu, MDM2-regulated degradation of HIPK2 prevents p53Ser46 phosphorylation and DNA damage-induced apoptosis, Mol. Cell 25 (2007) 739–750.
[50] D.W. Choi, Y.M. Seo, E.A. Kim, K.S. Sung, J.W. Ahn, S.J. Park, S.R. Lee, C.Y. Choi, Ubiquitination and degradation of homeodomain-interacting protein kinase 2 by WD40 repeat/SOCS box protein WSB-1, J. Biol. Chem. 283 (2008) 4682–4689.
[51] M. Winter, D. Sombroek, I. Dauth, J. Moehlenbrink, K. Scheuermann, J. Crone, T.G. Hofmann, Control of HIPK2 stability by ubiquitin ligase Siah-1 and checkpoint kinases ATM and ATR, Nat. Cell Biol. 10 (2008) 812–824.
[52] M.A. Powers, M.M. Fay, R.E. Factor, A.L. Welm, K.S. Ullman, Protein arginine methyltransferase 5 accelerates tumor growth by arginine methylation of the tumor suppressor programmed cell death 4, Cancer Res. 71 (2011) 5579–5587.
[53] O. Fehler, P. Singh, A. Haas, D. Ulrich, J.P. Müller, J. Ohnheiser, K.-H. Klempnauer, An evolutionarily conserved interaction of tumor suppressor protein Pdcd4 with the poly(A)-binding protein contributes to translation suppression by Pdcd4, Nucleic Acids Res. 42 (2014) 11107–11118.
[54] G.M. Pierantoni, F. Esposito, M. Tornincasa, C. Rinaldo, G. Viglietto, S. Soddu, A. Fusco, Homeodomain-interacting protein kinase-2 stabilizes p27kip1 by its phosphorylation at serine 10 and contributes to cell motility, J. Biol. Chem. 286 (2011) 29005–29013.
[55] J. Yu, H. Desmukh, R.J. Gutmann, R.J. Emnett, F.J. Rodriguez, M.A. Watson, R. Nagarajan, D.H. Gutmann, Alterations of BRAF and HIPK2 loci predominate in sporadic pilocytic astrocytoma, Neurology 73 (2009) 1526–1531.
[56] H. Desmukh, T.H. Yeh, J. Yu, M.K. Sharma, A. Perry, J.R. Leonard, M.A. Watson, D.H. Gutmann, R. Nagarajan, High-resolution, dual-platform aCGH analysis reveals frequent HIPK2 amplification and increased expression in pilocytic astrocytomas, Oncogene 27 (2008) 4745–4751.
[57] Y. Cheng, M.A. Al-Beiti, J. Wang, G. Wei, J. Li, S. Liang, X. Lu, Correlation between homeodomain-interacting protein kinase 2 and apoptosis in cervical cancer, Mol. Med. Rep. 5 (2012) 1251–1255.
[58] G. Defteros, S.R. Corrie, Q. Feng, J. Morihara, J. Stern, S.E. Hawes, N.B. Kiviat, Expression of mir-21 and mir-143 in cervical specimens ranging from histiologically normal through to invasive cervical cancer, Plos One 6 (2011) e28423.